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Review

Cbl signaling networks in the regulation of cell function

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Abstract. Cbl proteins control multiple cellular processes by acting as ubiquitin ligases and multifunctional adaptor molecules. They are involved in the control of cell proliferation, differentiation and cell morphology, as well as in pathologies such as autoimmune diseases, inflammation and cancer. Here we review recent advances in understanding the role of Cbl and the importance of a growing repertoire of Cbl-interacting proteins in the regulation of signaling pathways triggered by growth factors, antigens, cell adhesion, cytokines and hormones. We also address key issues of the nature of proteins that bind Cbl in particular cells, where they are located, and how they are altered or traffic within cells upon stimulation. It is becoming obvious that temporal and spatial changes in Cbl signaling networks are essential for the control of physiological processes in a variety of cells and organs and that their deregulation can result in the development of human diseases.

Key words. Cbl; signaling network; receptor tyrosine kinases; ubiquitination; CIN85; insulin; immune response.

Introduction

Over the last years we have begun to appreciate the complexity of signaling networks by which cell surface receptors convert external signals into biological responses. Even a short glimpse at the scientific literature reveals the vast information about signaling pathways present in the cell. At the same time, it is becoming clear that important biological issues, such as how cells grow, move, divide and die, will require detailed molecular insights into the cell's interior, where the signaling pathways convey messages that eventually control physiological or pathological processes. The recent start of the Alliance for Cellular Signaling (AfCS), a collaborative network of around 50 scientific groups [1], gives a timely and much needed lead on how to study intracellular signaling networks. This approach will involve the collection and assembly of thousands of mini-networks linked to individual signaling molecules and their dynamic changes at a given time and space in the cell. The end result may be a virtual cell with its complete dynamic complexity.

In this review we discuss cellular signaling from a single molecule perspective, focusing on the Cbl protein family. Cbl, like many signaling proteins, plays multiple and sometimes opposing roles in the regulation of signal transduction in different cells and in response to different stimuli. For example, Cbl attenuates signaling from tyrosine kinases by mediating their ubiquitination and downregulation. On the other hand, Cbl contributes to the formation of protein complexes implicated in osteoclast function, cell adhesion and insulin-dependent glucose transport. Furthermore, the ubiquitin ligase activity and adapting functions of Cbl also act in concert to promote certain cellular pathways, e.g. endocytosis of transmembrane receptors. The analysis of mutant mice lacking members of the Cbl family has indicated an increased complexity of molecular mechanisms by which Cbl signaling networks control diverse physiological responses in vivo. Furthermore, pathological changes in Cbl signaling pathways have been associated with the development of human diseases.

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The Cbl family of adaptor proteins with ubiquitin ligase activity

Cbl is a member of a protein family that is structurally and functionally conserved in multicellular organisms. Intensive research on Cbl proteins originates from the identification of the v-Cbl oncogene (for Casitas B-lineage lymphoma), which is transduced by the mouse Cas NS-1 retrovirus and induces pre- and pro-B lymphomas and fibroblast transformation [2]. v-Cbl encodes the amino terminal part (human Cbl residues 1–357) of the mammalian c-Cbl gene product (Cbl) that does not possess transforming capabilities [3]. Cbl is a 120-kDa cytoplasmic protein that is ubiquitously expressed with levels being highest in thymus and testis [4]. In mammalian cells, two related members of the Cbl family, Cbl-b and Cbl-c, are also present (fig. 1). Cbl-b, which is the most closely related to Cbl, is found in numerous cell types and is highly expressed in mature T cells [5]. In contrast, Cbl-c, a shorter homologue of Cbl also known as Cbl-3, is enriched in endodermally derived organs, but expressed at low levels in hematopoietic tissues [6–8]. Cbl orthologues in chicken, *Xenopus*, *Drosophila melanogaster* (long D-CblL and short D-CblS) and *Caenorhabditis elegans* (SLI-1) have also been identified [9–12]. Comparison of their sequence homology indicated a high degree of conservation in the amino terminal parts, which are composed of the phosphotyrosine binding (PTB) domain, a short linker region and the RING finger domain (fig.1). The PTB domain, which was also named the TKB (tyrosine kinase binding) domain (residues 50–350), is composed of three different subdomains: a four-helix bundle (4H), a calcium-binding EF hand and a divergent Src homology 2 domain (SH2) [13]. The zinc-binding C3HC4 RING finger domain (residues 365–428) recruits ubiquitin-conjugating enzymes (E2, Ubc) and mediates the transfer of ubiquitin onto a target protein [14]. Both the crystal structure and biological data [15] revealed that the linker region participates in the correct positioning of the PTB domain toward the RING finger domain and stabilizes their spatial orientation with the substrate and the E2 enzyme necessary for the efficient transfer of ubiquitin. Based on the conserved structure of their amino terminal parts, all members of the Cbl family possess the double property of recognizing activated targets proteins and of mediating their ubiquitination, a characteristic that is crucial for their function in regulating signaling pathways [14–18]. The carboxyl terminal sequences of Cbl proteins are more divergent among the various members of the family. Cbl, Cbl-b, D-CblL and chicken Cbl have a long carboxyl terminus that contains a proline-rich region, an acidic box domain with serine/threonine- and tyrosine-rich stretches and ends with a leucine zipper (LZ) domain with high homology to ubiquitin-associated (UBA) domain. In Cbl-c the carboxyl-terminal sequences are largely absent, thus

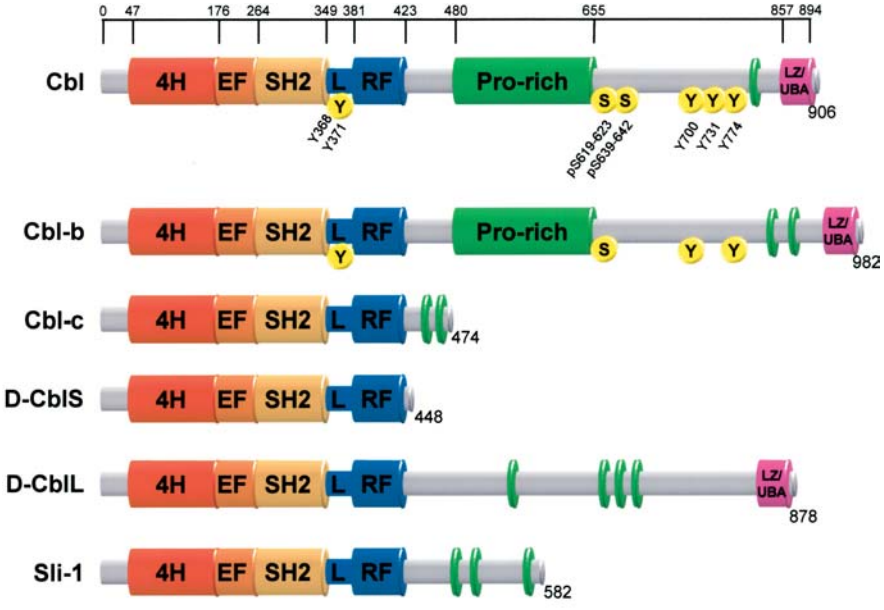

Figure 1. Members of the Cbl protein family. Domain structure and length of mammalian Cbl, Cbl-b and Cbl-c, *Drosophila melanogaster* D-Cbl short and long as well as *Caenorhabditis elegans* SLI-1 are shown. The amino-terminal PTB domain is composed of a four-helix bundle (4H), a calcium-binding EF hand and a divergent SH2 domain. The PTB domain, together with the linker region (L) and RING finger (RF), is the most conserved region in all Cbl proteins. The long Cbl homologues have an extended carboxyl-terminal part with proline-rich sequences (Pro-rich shown in green), several tyrosine and serine phosphorylation sites and a leucine zipper/ubiquitin-associated domain (LZ/UBA). The top scale indicates amino acid positions based on the human Cbl sequence.

closely resembling *C. elegans* SLI-1 and the *D. melanogaster*-Cbl short form [9, 10].

A repertoire of Cbl binding partners

Early after its discovery, Cbl was assumed to act as a multifunctional adaptor protein lacking any enzymatic activities. Biochemical studies in mammalian cells revealed that Cbl is rapidly phosphorylated upon numerous external or oncogenic signals and is able to associate with a range of intracellular signaling partners via interaction motifs and domains found in its carboxyl terminus. Subsequent findings have broadened this view and demonstrated that the PTB domain of Cbl directly binds to phosphorylated protein tyrosine kinases (PTKs), while the RING finger domain interacts with components of the ubiquitination machinery. Identification of novel Cbl-binding proteins further expanded the mechanisms by which Cbl participates in the regulation of signaling networks. The current list of Cbl-binding proteins is catalogued in table 1 and numbers around 40 partners, an amount that is likely to increase in the near future. The Cbl-interacting proteins are classified according to their ability to interact with particular domains and motifs in the Cbl molecule.

The PTB domain of Cbl binds numerous protein tyrosine kinases and other cellular phosphorylated proteins. It represents a novel class of phosphotyrosine recognition domain, as revealed by the crystallographic studies of the PTB domain of Cbl in complex with a phosphorylated peptide from the ZAP-70 tyrosine kinase [13]. Detailed sequence analysis revealed that Cbl PTB domain preferentially recognizes the aspartic acid and asparagine/aspartic acid at positions –3 and –2 relative to the phosphorylated tyrosine 292 in human ZAP-70 (SDGpY^{292}TPEP) [19]. Cbl also binds the analogous sequence in the Syk kinase (FNPpY^{323}EPEL) [20]. Almost all members of the Src family possess the Cbl PTB domain binding consensus motif (DNEY) at their autophosphorylation site, and Cbl binds directly to the phosphorylated Y416 in the activation loop of Src [21]. Other studies revealed the importance of a hydrophobic amino acid at the +4 position from phosphotyrosine in the preferential binding site of the Cbl PTB domain [13, 22]. Studies in *C. elegans* on binding between SLI-1 and LET-23, identified the sequence (SSRpY^{1225}KTEP) in the carboxyl terminal tail of LET-23 that mediates the negative effect of SLI-1 [23]. In the case of the epidermal growth factor receptor (EGFR), the major Cbl PTB binding site has been mapped to the phosphorylated tyrosine Y1045 (LQRpY^{1045}SSDP) [22]. Cbl's PTB interacting motifs found in other PTKs resemble the conserved pYXXØ sequence, e.g. pY973 of CSF-1 receptor (PNNpY^{973}QFC) [24] and pY1003 of HGF receptor c-Met (SVDpY^{1003}RATF) [25]. G306E mutation in Cbl's PTB domain abrogates its associations with the putative binding motifs [26, 27]. In addition to PTK's several adaptor proteins, known to be phosphorylated in mammalian cells, have been identified as targets of the PTB domain of Cbl. Cbl binds to the B-cell linker protein (BLNK) at the putative binding motif (DDSpY^{115}EPPP) in the carboxyl terminus of BLNK [28]. In addition, Cbl can directly interact with pY618 of APS, an adaptor protein phosphorylated in insulin-stimulated cells [29]. Recently, the PTB domain of Cbl was shown to bind directly to Y55 of Spry2 that contains the conserved TNEpY^{55}TEGP motif [30].

The RING finger domain of Cbl interacts with E2 enzyme (UbcH7) in vivo and in vitro [15, 18]. More recently, Sprouty2 proteins (mSpry2 and dSpry2), have been shown to interact with the RING finger of Cbl and D-Cbl, but not with other RING finger-containing proteins [31, 32]. It seems that the binding surface on the RING domain is shared between Spry2 and UbcH7 since they can effectively compete with each other for binding to Cbl [31].

The majority of identified Cbl partners associate with motifs and domains present in the carboxyl terminal half of Cbl. Poly-proline sequences are largely clustered in a proline-rich region adjacent to the RING domain, while a few motifs are scattered throughout the distal carboxyl terminus of long forms of Cbl. The proline-based sequences of Cbl are implicated in interactions with SH3 or WW domain-containing proteins. The adaptor protein Grb2 is constitutively associated with Cbl via its amino-terminal SH3 domain [33]. The Grb2 binding site was mapped to the conserved poly-proline region (residues 481–563) in all mammalian Cbl proteins [34, 35]. This sequence is not present in long D-Cbl, which accordingly does not interact with Grb2/Drk [11]. Efficient binding of the adaptor molecule Nck to the proline-rich region of Cbl involves cooperation between the first and second SH3 domains of Nck [36, 37]. The SH3 domain of the p85 subunit of phosphatidyl inositol-3 (PI-3) kinase binds to the distal proline-rich sequences of Cbl-b (residues 595–982) [38, 39]. More recently, several adaptor proteins with three SH3 domains, belonging to the CAP/ArgBP2/ponsin and CIN85/CMS families were found to bind to Cbl and Cbl-b [40–44]. The carboxyl terminal SH3 domain of CAP (Cbl-associated protein) binds constitutively to the proline-rich region of Cbl [40], while ArgBP2 associates with two poly-proline sequences of Cbl via its second and third SH3 domains [41]. On the other hand, Cbl interactions with members of the CIN85/CMS protein family are increased by growth factor stimulation [43, 44]. CIN85 (Cbl-interacting protein of 85 kDa) and CMS (Cas ligand with multiple SH3 domains) bind to the distal carboxyl-terminus of Cbl, recognizing a novel proline-arginine motif present in Cbl and Cbl-b, but not in Cbl-c [42]. It was postulated that

Table 1. Current repertoire of Cbl-binding proteins is listed according to Cbl motifs and domains involved in interactions. The known interaction of Cbl-b and Cbl-c with these proteins is indicated ('+' interaction; '–' no-interaction). The functional role of Cbl-protein complex is indicated whenever determined. Details of their interactions together with references are provided in the text. N.D. (not determined).

Protein	Protein domain	Cbl domain	Cbl-b	Cbl-c	Functional role
EGFR	pY-1045	PTB	+	+	Ligand-dependent receptor downregulation
ErbB-2	pY-1112	PTB			Ligand-dependent receptor downregulation
HGFR	pY-1003	PTB			Ligand-dependent receptor downregulation
CSF-1R	pY-973	PTB			Ligand-dependent receptor downregulation
ZAP-70	pY-292	PTB	+		Inactivation of ZAP-70, regulation of TCR signaling
Syk	pY-323	PTB	+		Inactivation of Syk, regulation of BCR and FcR signaling
Src	pY-416	PTB			Inhibition of Src activity
BLNK	pY-115	PTB	+		Negative regulation of BLNK in BCR signaling
Sprouty2	pY-55	PTB			Competition of Cbl binding to EGFR
APS	pY-618	PTB			Recruitment of Cbl to activated insulin receptor, c-kit, PDGFR
Lyp1	Putative PXXpY	(PTB)			Dephosphorylation of Cbl
Sprouty2	C-term domain	RING finger			Inhibition of RTK ubiquitination
UbcH7	L1 and L2 loops	RING finger			Ubiquitination of proteins
Grb2	SH3	pro-rich (481–563)	+	–	Indirect recruitment of Cbl to EGFR
Nck	SH3 A & B	pro-rich & 820–828	(+)		Regulation of Xenopus embryo patterning
p85 (PI-3 kinase)	SH3	pro-rich (595–982 in Cbl-b)	+		Ubiquitinaiton and negative regulation of PI-3 kinase, cell morphology
CAP	SH3	pro-rich	+		GLUT4 translocation, actin regulation
ArgBP2	SH3 B & C	pro-rich			Negative regulation of Abl kinase by Cbl
CIN85	SH3 A,B,C	pro-arg motif	+	–	RTK endocytosis
CMS	SH3 A,B,C	pro-arg motif	+		Cell morphology
PLCγ*1/2*	SH3	pro-rich	+	–	Calcium levels in TCR/BCR pathways
Fyn	SH3	pro-rich (552–614)	+	+	TCR signaling
Src, Fyn, Lck, Hck	SH3	pro-rich			Osteoclasts functions, RTK downregulation, cell morphology, ubiquitination and degradation
Fgr	SH3	pro-rich	+	–	Ubiquitination
Lyn	SH3	pro-rich	+	+	Signaling from B cell, HGF, G-CSF receptors
Btk	SH3	pro-rich (526–537)	+		BCR signaling
Abl	SH3	pro-rich			Cytoskeleton regulation (involve PI3K, CrkL)
AIP4	WW	pro-rich		+	Downregulation of activated RTKs
Myosin 1C	SH3	pro-rich			N.D.
SH3P17	SH3	pro-rich			Regulation of endocytosis
Dap160	SH3	N.D.			Endocytosis
CrkL, Crk II, I	SH2	pY-700/774	+		C3G/Rap1 regulation, Cell morphology
Vav	SH2	pY-700	+		TCR signaling
p85 (PI-3 kinase)	SH2	pY-731	(+)		Negative regulation of PI-3 kinase activity, cell morphology
SFKs	SH2	N.D.			Stabilization of interactions with Cbl
Abl	SH2	N.D.			Stabilization of the complex with Cbl
PKC α/θ	N.D.	C-terminal Cbl			Inhibition of Cbl tyrosine phosphorylation
14-3-3	N.D.	pS-619-623 and pS-639-642	–		N.D.
Cbl	leucine zipper	leucine zipper			Homodimerization
SLAP2	C-term part	N-term part	+		Inhibition of TCR signaling

phosphorylation of Cbl promotes a conformational change in the carboxyl terminus of Cbl, leading to exposure of the CIN85-SH3-domain binding site [41, 43, 44]. Similarly, Cbl forms a complex with the SH3 domain of PLC-γ1 in an epidermal growth factor (EGF)-dependent manner [45].

Members of the Src family kinases (SFKs) can also associate with Cbl via their SH3 domains. The SH3 domain of Fyn is constitutively associated with a region encompassing residues 552–614 of human Cbl [46]. Cbl shows preferential in vitro binding to the SH3 domain of Fyn when compared with Lck's SH3 domain [47, 48]. It has been proposed that Cbl is primarily associated with Fyn in T cells and with Lyn in B cells [47, 49]. Upon tyrosine phosphorylation of Cbl, a more stable interaction with SFKs is established via SH2 domain binding [47]. In contrast, Src-related Bruton's tyrosine kinase (Btk) binds less efficiently to phosphorylated Cbl [50]. The SH3 domain of the Abl PTK directly associates with proline-rich motifs in Cbl, and this interaction has been reported to open the protein conformation and activate Abl kinase. Subsequently, the SH2 domain of Abl binds to phosphorylated tyrosine residues in Cbl and thus stabilizes the Cbl/Abl complex [51].

Cbl-c has a limited set of interacting partners due to minimal carboxyl-terminal sequences. Cbl-c associates with Fyn [7], SH3 domains of Lyn and Crk [8], and the pon-sin/CAP/vinexin protein family [52]. More recently, interactions between Cbl-c and AIP4/ITCH E3 ligase, which belongs to the HECT family of E3 ligases, has been detected. The binding was mediated via the WW domains of AIP4 and the proline region of Cbl-c and Cbl [52].

Cbl contains 22 tyrosine residues, some of which are phosphorylated in response to external stimuli [48, 53]. Tyrosines 700, 731 and 774 are the major phosphorylation sites in vivo and mediate interactions with diverse SH2 domain-containing signaling proteins [54]. The SH2 domains of adaptors Crk and Crk-L bind to phosphorylated Y700 and Y774 residues in Cbl and to the corresponding sequence in Cbl-b [39, 55]. The SH2 domain of Vav, the Rac/Rho guanine nucleotide-exchange factor, binds phosphotyrosine 700 of Cbl after stimulation of T cell receptors [56]. The entire SH3-SH2-SH3 region of Vav contributes to binding to Cbl-b [57], suggesting involvement of the SH3 domains in this interaction. Following stimulation of immune receptors and Cbl phosphorylation at Y731, the SH2 domain of the p85 subunit of PI-3 kinase associates with Cbl [54]. The SH2 domain-containing tyrosine phosphatase SHP2 was also found to bind to phosphorylated Cbl [58].

The 14-3-3 adaptor molecules recognize two tandem serine motifs in the carboxyl terminus of Cbl ($R^{617}H\underline{S}LPF\underline{S}$ and $R^{636}LG\underline{S}TF\underline{S}$), which are phosphorylated by protein kinase C following T-cell-receptor stimulation [59]. Since both motifs are necessary for efficient association, 14-3-3 does not bind Cbl-b, which lacks the second site [59].

The leucine zipper domain at the carboxyl terminus of Cbl and Cbl-b is involved in homotypic interactions, thus promoting homodimerization of Cbl molecules [60]. The sequence coding for the predicted leucine zipper structure of Cbl coincides with a consensus sequence for UBA domain, which is found in multiple components of the ubiquitination pathway [61] and is known to bind polyubiquitin chains [62]. Yet, the UBA domain of Cbl has not been shown to bind directly to ubiquitin.

It is becoming evident that multiple direct and indirect interactions between Cbl and many signaling proteins are detected in vivo following cell stimulation. The functional significance of large protein complexes assembled around Cbl will be elaborated in the following sections.

Cbl as a ubiquitin ligase

Ubiquitination is a reversible modification of cellular proteins where a single ubiquitin (monoubiquitination) or a chain of ubiquitins (polyubiquitination) is attached to a target protein [63, 64]. Polyubiquitination of proteins has long been recognized as an evolutionary conserved modification required for protein degradation by the proteasome. On the other hand, monoubiquitination appears to control nonproteolytical functions such as endocytosis of cell membrane receptors, virus budding, histone activity and DNA repair [64]. The process of recognition and transfer of ubiquitin to target proteins involves a cascade of ubiquitin-modifying enzymes. Ubiquitin, initially synthesized in a polymeric form, is cleaved into monomers that are activated by carboxyl terminal adenylation and attachment to the E1 (ubiquitin-activating) enzyme [63]. Activated ubiquitin is subsequently transferred to one of the ubiquitin-conjugating enzymes (E2). The E2 transfers ubiquitin either to an E3 ubiquitin ligase (as in the case of HECT domain E3s), or directly to a lysine of target proteins with the assistance of RING-type E3 ubiquitin ligases, such as Cbl [63].

The first indications that Cbl is involved in ubiquitination processes came from studies on signaling pathways mediated by the colony stimulating factor-1 receptor (CSF-1R) in macrophages [65]. Upon CSF-1 stimulation, Cbl is recruited to the receptor and rapidly ubiquitinates the activated CSF-1R [65]. The following reports demonstrated that Cbl overexpression enhances ubiquitination of EGF, PDGF and CSF-1 receptors and leads to increased degradation of the activated receptors [66–68]. The importance of Cbl as an E3 ligase has been elucidated with the discovery that the isolated RING finger of Cbl can catalyze the addition of ubiquitin moieties to a target protein in vitro and in vivo [14, 16]. Mutation of the critical residue cysteine 381 (C381A) disrupts the association of

Cbl with the E2, rendering Cbl protein nonfunctional despite the unaltered PTB domain [14, 16]. Similarly, mutation of tryptophan W408, a residue conserved among all RING-type E3s, disrupts the ubiquitin ligase function of Cbl [14, 16]. Solving the three-dimensional structure of Cbl bound to the E2 enzyme UbcH7 and a phosphorylated ZAP-70 peptide revealed that the amino terminal part of Cbl is critical to ensure high-affinity interactions and local increase in concentration between substrates and E2, thus efficiently facilitating ubiquitin transfer [15].

In addition, there have been reports showing that Cbl can mediate ubiquitination of proteins bound to its carboxyl terminal region rather than to the PTB domain. Such examples include the p85 subunit of PI-3 kinase [38] and the CIN85/CMS protein family [69]. Cbl-b overexpression stimulates the ubiquitination of p85, which is dependent on a functional Cbl-b RING finger, and an interaction between the p85 SH3 domain and the carboxyl terminus of Cbl-b [38]. Both Cbl and Cbl-b can mediate EGF-induced monoubiquitination of CIN85 [69], which also requires an intact RING finger and a CIN85 binding motif in the distal carboxyl terminus of Cbl or Cbl-b [69]. Physical and functional interactions between Cbl and other types of ubiquitin ligases have also been reported. In mammalian cells, Cbl predominantly ubiquitinates RTKs and closely associated proteins such as CIN85 [69], while Nedd4, a HECT type E3, is involved in ubiquitination of Eps15, an endocytic regulatory protein required for sorting of EGF receptors in the endosome [70]. Following immunoglobulin engagement, Cbl and Nedd4 are copartitioned into lipid rafts where they may ubiquitinate components of receptor complexes [71]. Association between Cbl-c and AIP4, another member of HECT-type E3 ubiquitin ligase, is also important for EGFR downregulation [52]. Overexpression of isolated WW domains of AIP4 resulted in a decrease of Cbl-c-mediated EGFR ubiquitination and in upregulation of EGF-induced gene transcription. It is possible that Cbl cooperates with other E3 ligases, directly or sequentially, leading to a compartment-specific regulation of various signal transduction pathways. Clearly, more studies are needed before we fully understand the role of Cbl as a ubiquitin ligase as well as the relevance of ubiquitination of various Cbl targets in mammalian cells.

Dual role of Cbl in regulation of RTK endocytosis

Ligand-mediated endocytosis of RTKs begins with the rapid recruitment of activated receptors to clathrin-coated pits, which eventually pinch off and form clathrin-coated

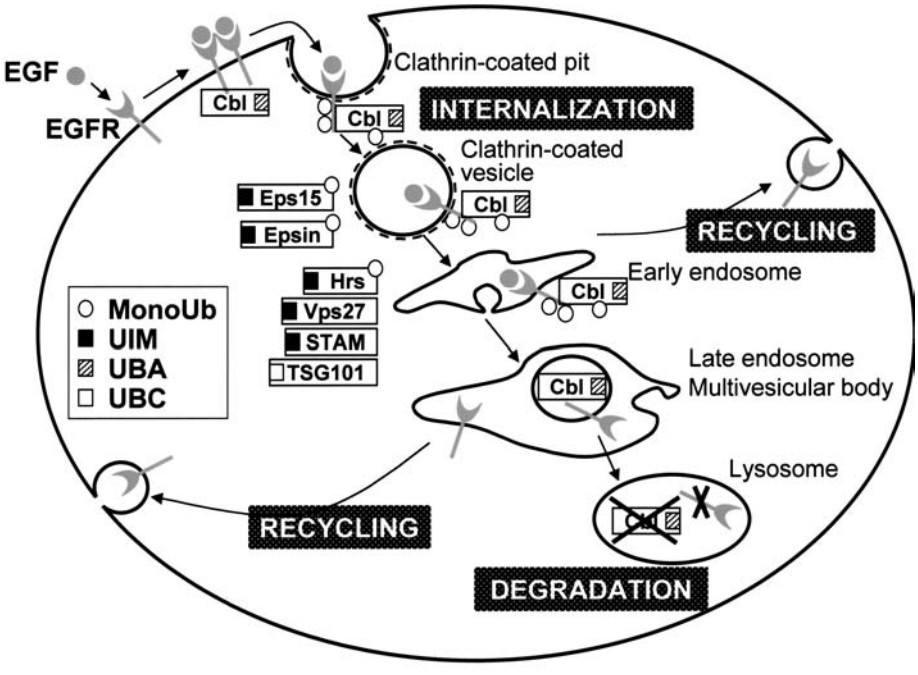

Figure 2. Intracellular trafficking of activated receptors. The activation of EGF receptors by EGF induces their phosphorylation and the recruitment of Cbl that mediates receptor multiubiquitination. This process is rapidly followed by the clathrin-dependent internalization of activated receptors/Cbl complexes and their subsenquent trafficking towards various intracellular compartments leading, finally, to either recycling of receptors to the cell surface or their concomitant degradation in the lysosome. These mechanisms are regulated by diverse proteins able to interact with ubiquitin moieties via specific domains (UIM, Ubiquitin Interacting Motif; UBA, Ubiquitin Associated; UBC, Ubiquitin Conjugating enzyme like) and that can also undergo monoubiquitination.

vesicles that mature into early endosomes. Subsequent membrane fusions and protein-sorting processes direct the trafficking of the receptors throughout the endocytic compartments, finally resulting in proteolytic degradation in lysosomes (fig. 2). These processes require the kinase activity of RTKs, interactions with multiple endocytic proteins and continuous attachment of ubiquitin to a traveling cargo [72–74]. Recent identification and functional characterization of a variety of ubiquitin-binding domains that are present in proteins linking internalized RTK receptors with the endocytic machinery have provided strong evidence for the essential role of ubiquitination in membrane-trafficking processes from yeast to mammals [61, 70, 75]. The importance of these findings, shown schematically in figure 2, has been reviewed in more details elsewhere [63, 64, 76].

Monoubiquitination and RTK endocytosis

Since Cbl plays a pivotal role in mediating ligand-induced ubiquitination of RTKs, it has been assumed that Cbl controls processes of receptor internalization, trafficking and degradation. Cbl has been shown to associate with and to ubiquitinate the EGFR at the cell membrane, under conditions where endocytosis was impaired [77, 78]. Similarly, upon CSF-1 stimulation Cbl is targeted to the plasma membrane [65] and promotes rapid internalization of the CSF-1R [68]. Cbl remains associated with RTKs in early and late endosomes [66, 78], which enables Cbl to progressively ubiquitinate receptors and thus ensures receptor sorting for degradation. Similarly, Cbl-b is sorted together with EGFR in the endosome and undergoes common degradation in the lysosome [79]. Despite a large number of studies on ubiquitination of RTKs, it remains to be determined whether receptor ubiquitination represents a critical signal for endocytosis or is only a modulator of this process. It has generally been described that polyubiquitination of RTKs occurs in vivo, based on the observed smeary patterns of ubiquitinated RTKs in SDS-polyacrylamide gel electrophoresis (PAGE) gels resembling polyubiquitinated cytoplasmic proteins. This is, however, contradictory to current understanding that polyubiquitination of proteins targets them for destruction by the proteasome, which is a distinct pathway from lysosomal degradation of RTKs [80]. Furthermore, studies in yeast indicated that monoubiquitination is sufficient for transmembrane receptor internalization [81]. In accordance with the latter, recent studies have indicated that Cbl directs monoubiquitination, rather than polyubiquitination, of activated EGF and PDGF receptors in mammalian cells [82]. RTKs are monubiquitinated on multiple sites, which ensures proper endosomal sorting and subsequent degradation of receptors in the lysosome [82]. Furthermore, a single ubiquitin attached to EGF or transferrin receptors was sufficient to mediate internalization as well as degradation of receptors [82, 83]. These results are consistent with the hypothesis that a single ubiquitin carries intrinsic signals for internalization at the plasma membrane and sorting for lysosomal destruction.

Cbl-binding proteins and RTK endocytosis

A significant body of evidence has recently converged to indicate that Cbl regulates RTK endocytosis via pathways that are functionally separable from its ubiquitin ligase activity and are dependent on Cbl's ability to interact with multiple proteins in the endocytic compartment. Many of these molecules are implicated in the control of receptor trafficking, thus positioning Cbl as a link between RTKs and the endocytic regulatory network. The kinase activity of Src, which interacts via its SH3 domain with Cbl, has been implicated in EGFR internalization via clathrin-coated pits (reviewed in [84]). Src binds, phosphorylates and activates dynamin [85] and phosphorylates the heavy chain of clathrin [86]. Inhibition of Src activity delays internalization of activated receptors [86]. Studies on PDGFRα have also suggested a cooperative action of Cbl and Src in receptor downregulation [87]. Dap160, a *D. melanogaster* adaptor protein with the Eps15 homology (EH) domain and four consecutive SH3 domains that associate with synaptojanin and synapsin [88], simultaneously associates with dynamin and D-CblL [12]. In addition, SH3P17, a four SH3 domain-containing protein homologous to Dap160 and intersectin, bound to the proline-rich region of Cbl in a yeast two-hybrid screen [89]. Cbl also associates with the SH3 domain-containing human unconventional myosins [89] that are involved in vesicle transport and receptor-mediated endocytosis in *Saccharomyces cervisiae* [90] and mammals [91]. In addition, PI-3 kinase associated with Cbl may regulate membrane trafficking and endosome recycling by modulating local levels of phosphoinositides (reviewed in [92]).

More recently, Cbl and Cbl-b were shown to regulate RTK internalization by binding to the adaptor protein CIN85 [42, 93, 94]. Interaction between CIN85 and Cbl/Cbl-b is increased following tyrosine phosphorylation of Cbl [42], while CIN85 constitutively associates with endophilins, a family of proteins able to control plasma membrane invagination during internalization [95, 96]. In that way CIN85 recruits endophilins in the complex with activated RTKs, thus controlling early steps in receptor endocytosis. Accordingly, inhibition of Cbl-CIN85-endophilin interactions was sufficient to block RTK endocytosis and degradation, without perturbing the ability of Cbl to ubiquitinate activated receptors [93, 94]. In addition, CIN85 colocalizes with Cbl and EGFRs in the endosome and is monoubiquitinated by Cbl and Cbl-b in these complexes [69]. CIN85 also binds to

numerous regulatory proteins in the endosome and was recently proposed to function as a scaffolding molecule able to control endocytosis of RTKs [97].

Cbl thus plays a dual role in RTK endocytosis by linking receptors with the endocytic machinery and by promoting ubiquitination of RTKs and receptor-associated proteins. In view of an increasing repertoire of Cbl-interacting partners and emerging evidence for the relationship between ubiquitination and internalization, we probably still underestimate the functional role of Cbl in multiple steps of RTK endocytosis.

Cbl in regulation of PTK signaling

There are numerous studies demonstrating that Cbl proteins regulate, both positively and negatively, signaling by PTKs [73]. On the one hand, Cbl is rapidly tyrosine phosphorylated following activation of multiple PTKs and is involved in forming large signaling networks around activated PTKs (fig. 3A). On the other hand, genetic evidence in *C. elegans* [9] and *D. melanogaster* [11], and biochemical studies in mammalian cells, have pointed to the major role of Cbl in the inhibition of PTK signaling by mediating their ubiquitination and degradation (fig. 3B). Even though these may be the prevailing functions

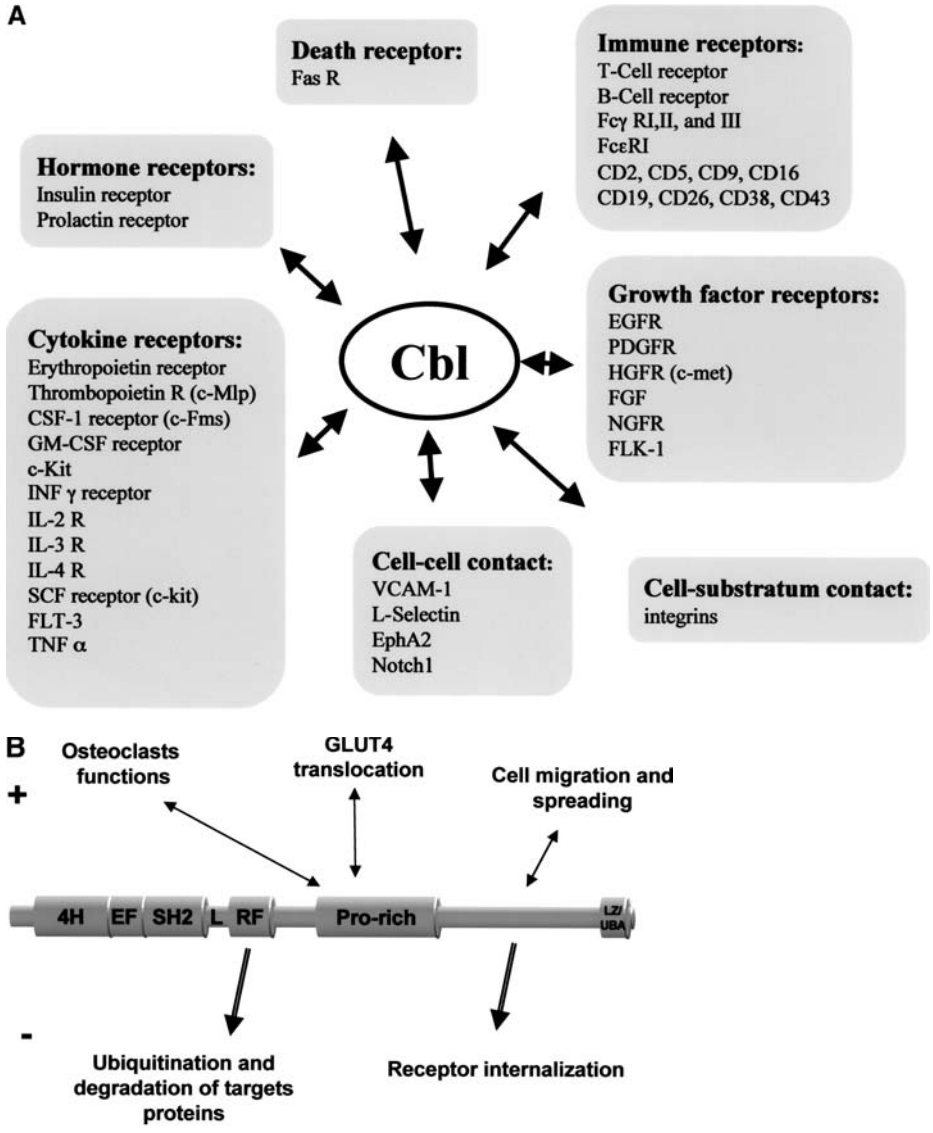

Figure 3. (*A*) Cbl participates in signaling networks that originate from various types of receptors. (*B*) Cbl's role in positive and negative signaling. Multiple Cbl domains participate in induced or constitutive binding to a large set of signaling proteins. Depending on the content and localization of complexes, Cbl is involved in both transmission and inhibition of signals, often in the same cellular context. Examples of positive Cbl signaling include regulation of cell migration and spreading, osteoclast functions and glucose transporter translocation. On the other hand, Cbl association with CIN85 leads to internalization of RTKs, which together with Cbl's E3 ubiquitin ligase activity, regulates receptor endocytosis and degradation.

of Cbl proteins in regulation of PTK signaling, the molecular details of their actions as well as their biological significance vary depending on the type of tyrosine kinase. For example, RTKs are monoubiquitinated on multiple sites and degraded in the lysosome [82], while Src kinases are polyubiquitinated and degraded in the proteasome [98] (fig. 4). Furthermore, interactions of Cbl with distinct PTKs vary in different cells and in response to different stimuli. In the following sections we will discuss the current knowledge of specific Cbl actions in modulating signaling by individual members of receptor and non-receptor-type PTKs.

ErbB receptor family

Each of the four members of the ErbB RTK family has specific sets of stimulatory ligands and associated signaling proteins, which together with their ability to form homo- and heterodimers contributes to diverse cellular responses. Cbl associates preferentially with EGFR (ErbB-1) and to a less extent with ErbB-2 [99]. Upon binding of EGF, EGFR is rapidly internalized and sorted to lysosomal degradation, while stimulation with transforming growth factor α (TGFα) results in increased receptor recycling [74]. Following ligand stimulation, Cbl associates with EGFR at the plasma membrane and is colocalized with the internalized receptors in the endocytic compartments [78]. The lysosomal targeting motif, found in the carboxyl-terminal sequence of EGFR, contains the Cbl-binding site, i.e. tyrosine 1045 [100]. Interestingly, EGFR activation by exposure to oxidative stress (H_2O_2), which fails to induce phosphorylation of the Y1045 residue, does not lead to Cbl-mediated receptor ubiquitination or internalization [101]. The fact that Cbl cannot interact with ErbB-3 and ErbB-4 corresponds to the impaired internalization of these receptors upon ligand stimulation [99]. The downregulatory role of Cbl in ErbB receptor signaling has been extensively studied in a variety of cell systems (i.e. vulval development in *C. elegans* and R7 photoreceptor neuron development in a *Drosophila*). The negative regulation of RTKs by Cbl was first identified in *C. elegans*, where the disruption of Cbl (SLI-1) activity can compensate for a decreased activity of LET-23 [23]. Finally, the importance of the regulation of EGFR activity by Cbl is emphasized by the fact that disturbances in Cbl function are linked to ErbB receptor-dependent tumor formation (see below).

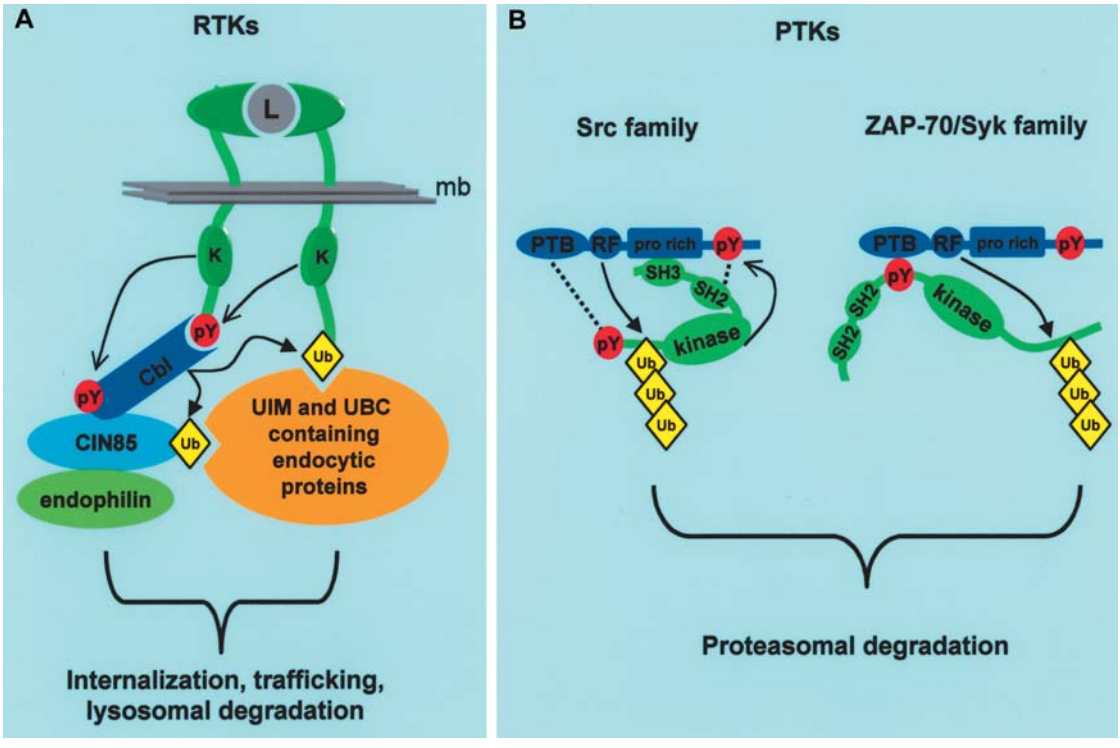

Figure 4. Dual function of Cbl in downregulation of protein tyrosine kinases. (*A*) Ligand (L)-induced dimerization and autophosphorylation of RTKs leads to Cbl binding to phosphotyrosine (pY) motifs at the carboxyl-terminal tail of the receptor. Phosphorylation of Cbl by RTKs recruits CIN85/endophilin in the complex with activated RTKs. Cbl also mediates monoubiquitination of RTKs and CIN85 that is essential for targeting the receptor complex for lysosomal degradation via UIM- and UBC-containing endocytic proteins. (*B*) Cbl downregulates cytoplasmic PTKs. Src kinases associate with Cbl proline-rich domain, while ZAP-70 and Syk interact with the Cbl PTB domain leading to inactivation of their kinase activities. In these complexes Cbl mediates polyubiquitination of PTKs, leading to their proteasomal degradation.

Platelet-derived growth factor receptors (PDGFR)
Ligand-induced ubiquitination of PDGFRβ plays predominantly a negative regulatory role on receptor mitogenic signaling [102]. Cbl overexpression in fibroblasts results in enhancement of ubiquitination and degradation of PDGFRα and PDGFRβ [67, 103], leading to reduction of PDGF-induced cell proliferation and protection against apoptosis. Interestingly, the Cbl-G306E mutant physically associates with activated PDGFRα, probably via Grb2 or Src. However, this interaction was not sufficient for receptor downregulation [103]. Accordingly, wild-type Cbl, but not the G306E mutant, inhibited PDGF-induced DNA synthesis [104]. Src kinases, which associate with phosphorylated PDGFRα, cooperate with Cbl in decreasing the half-life of the receptor as well as PDGF-induced DNA synthesis [87]. Additionally, studies on involvement of APS (adapter protein containing PH and SH2 domains) in PDGFR signaling indicated that Cbl associates with phosphorylated APS and that both proteins act synergistically in the inhibition of PDGF-induced c-fos activation [105].

Colony-stimulating factor-1 (CSF-1) receptor
Cbl interaction with the CSF-1 receptor participates in both negative and positive signaling. Direct Cbl binding to phosphotyrosine Y977 in the feline CSF-1 receptor results in rapid receptor ubiquitination and downregulation and reduced Erk activation in receptor-expressing fibroblasts [106]. Mutation Y977F abolishes both effects, even though Cbl association with the receptor is maintained, probably via PI-3 kinase [106]. The Cbl binding site is conserved in human CSF-1 receptor (Y973) [24], but is deleted in the transforming form of the receptor, v-Fms. According to another study, CSF-1 stimulation leads to recruitment of Crk-II and the p85 subunit of PI-3 kinase to phosphorylated Cbl and association of a multiprotein complex with the CSF-1 receptor via Grb2 [107].

Fibroblast growth factor receptors (FGFRs)
Binding of FGF together with heparan sulphate activates FGFRs and initiates intracellular signaling cascades that are predominantly mediated via Frs2 (FGF receptor substrate 2) adaptor proteins and phospholipase C gamma (PLCγ) [108]. Frs2 proteins are essential for the majority of biological responses elicited by FGF stimulation [109, 110]. However, it has recently become clear that Frs2 also coordinates negative regulatory signals necessary for termination of FGFR signaling [111]. One of the mechanisms involves the Grb2-dependent recruitment of Cbl in the complex with Frs2 and FGFR, followed by their subsequent ubiquitination and degradation [111]. Ligand-independent hyperactivation of mutated FGFR3 gives rise to various skeletal disorders [112]. While Cbl-mediated ubiquitination of FGFR3 is proportional to its intrinsic kinase activity, it fails to induce degradation of overactive mutants [113]. In addition, oncogenic FGFRs are internalization defective, despite high levels of phosphorylation and ubiquitination [114].

Hepatocyte growth factor receptor (HGFR, c-Met)
HGFR regulates B cell differentiation and controls motility and morphogenesis of epithelial cells. HGF stimulation induces a strong and transient phosphorylation of Cbl in B cells, and leads to an increased association of Cbl with Lyn, PI-3 kinase and CrkL [115]. Direct Cbl binding to the juxtamembrane region of the receptor (pY1003) is required for ligand-induced ubiquitination of the HGFR [25, 115]. The gain-of-function mutation of the Y1003 residue results in an increased scattering and fibroblast-like morphology of epithelial cells [116]. Finally, Cbl promotes HGFR internalization and degradation by forming an inducible complex with CIN85 and endophilins [94]. Cbl is also a positive effector downstream of the HGFR. Upon HGF-induced phosphorylation, Cbl interacts with the SH2 domains of Src, the p85 subunit of PI-3 kinase and Crk, while its association with the SH3 domain of Grb2 remains constant. Importantly, Cbl association with Crk leads to the activation of Rac and stimulation of the MAP kinase cascade [117].

KIT receptor
The hematopoietic growth factor c-kit ligand (KL), also called SCF (stem cell factor) or steel factor, is involved in the survival, expansion and differentiation of hematopoietic progenitor cells of various lineages. KL binding to the c-kit receptor activates the kinase activity of the receptor and stimulates the rapid tyrosine phosphorylation of Cbl [118]. The binding of Cbl to activated c-kit is indirect and mediated by adaptor proteins Grb2 [119] or APS [120]. Following stimulation by SCF, Cbl forms multiprotein complexes containing Grb2, CrkL and p85 of PI-3 kinase [121], and also mediates ubiquitination and downregulation of receptors. In addition, constitutively active receptor mutants found in human mastocytomas and gastrointestinal stromal tumors are constitutively associated with Cbl/Cbl-b and CIN85 [42].

Other receptor tyrosine kinases
The FLT3 RTK and its ligand, FL, play an important role in early hematopoietic development. Upon FL stimulation in myeloid cell lines, Cbl is phosphorylated and found in complex with p85 PI-3 kinase and CrkII [122]. Similarly, activation of FLT3 leads to tyrosine phosphorylation of Cbl-b in THP1 monocytic and JEA2 pro-B cells [123]. Vascular endothelial growth factor receptor-2 (Flk-1) associates with Cbl in response to shear stress and integrin-mediated cell adhesion [124]. Tyrosine phosphorylation of Cbl was also observed after stimulation with nerve growth factor (NGF) [34], insulin (see next section) and [33] following activation of EphA2 receptors [125].

Other transmembrane receptors

Cbl is involved in signaling by granulocyte-macrophage colony stimulating factor (GM-CSF) receptor as it is rapidly and transiently phosphorylated in lymphocytes stimulated with GM-SCF [33]. It also regulates the activity of the Notch receptor by inducing the ubiquitin-dependent lysosomal degradation of the phosphorylated receptor [126].

Protein tyrosine kinases ZAP-70/Syk

Cbl proteins are involved in the negative regulation of the ZAP-70/Syk PTK family in hematopoietic cells [127]. Following immunoreceptor activation, Cbl becomes tyrosine phosphorylated and, under conditions of overexpression, can associate with activated ZAP-70 and Syk kinases. The PTB and RING finger domains of Cbl are necessary and sufficient for Cbl-dependent inhibition of ZAP-70 and Syk [128]. The mechanism of inhibition is not yet completely clear, but studies from cell lines indicate it involves a direct binding of the Cbl PTB domain to the negative regulatory phosphorylation site within the region linking the SH2 and the catalytic domains in ZAP-70 (Y292) and Syk (Y323) [19, 20, 129]. Importantly, mutation of these critical tyrosines to phenylalanine results in gain-of-function phenotypes [130, 131]. However, a recent study of thymocytes from a mouse with a loss-of-function mutation in the Cbl PTB domain (i.e. G304E) did not show enhanced ZAP-70 activity [132]. This finding contrasts with the hyperactive ZAP-70 observed in Cbl knockout thymocytes and raises doubts about the role of the PTB domain in the negative regulation of ZAP-70. Overexpression of Cbl in the RBL-2H3 mast cell line reduces the autophosphorylation and kinase activity of co-expressed Syk and inhibits histamine release following FcεRI ligation [133]. In line with the observations from RTKs, the kinase activity of Syk is necessary for the Cbl-dependent ubiquitination of both Syk and FcεRI after antigen stimulation of RBL-2H3 cells [134]. Additionally, in Cbl-deficient thymocytes, phosphorylation of two main substrates of ZAP-70 kinase, SLP-76 and LAT, is increased and prolonged in comparison to normal cells [135]. However, in view of the fact that ZAP-70 levels are unaltered in the Cbl knockout thymocytes, the mechanism of negative regulation of ZAP-70 remains to be resolved.

Src family kinases (SFKs)

Src is a prototype for the family of PTKs containing amino terminal SH2 and SH3 domains followed by a catalytic domain and short carboxyl-terminal sequence. Src activity contributes to RTK and integrin signaling and is involved in multiple cellular processes, including adhesion, motility and DNA synthesis [136]. Cbl has been identified as a downstream substrate of multiple SFKs [137]. The ability of Cbl to block DNA synthesis by activated Src kinase is independent of its PTB and RING finger domain, but requires its carboxyl-terminal tail [104]. Furthermore, the PTB domain of Cbl can bind to phosphorylated Y416 in the activation loop of Src, thus inhibiting Src kinase activity and integrin-mediated adhesion [21]. Src kinase activity and tyrosine phosphorylation of Cbl are required for ubiquitination of Src and Cbl in vitro [138]. Similarly, Cbl-dependent ubiquitination and degradation were demonstrated for other active SFKs, Fyn [139], Lck [98] and Hck [140]. Interestingly, membrane-anchored Cbl potently induces ubiquitination and proteasomal degradation of kinase-inactive Hck [140]. On the other hand, active forms of Src and Blk are targets for ubiquitination mediated by a HECT domain E3 ligase [141]. Therefore, negative regulation of SFKs by ubiquitination may be controlled by both RING- and HECT-type ubiquitin ligases in vivo.

In addition to the negative regulation of SFKs, Cbl participates in positive signal transduction downstream of Src in the integrin-dependent pathways required for osteoclasts and macrophage functions [142–144]. Studies in Src-deficient osteoclasts indicated that Src was necessary for the tyrosine phosphorylation of Cbl and its normal localization in osteoclasts [142]. Cbl has been suggested to be involved in the process of bone resorption, as a downstream effector of Src in osteoclasts [142]. Furthermore, spreading and migration of macrophages in vivo are dependent on the SFK-mediated Cbl tyrosine phosphorylation and its associated PI-3 kinase activity [144].

Abelson kinase family

The Abl and Arg nonreceptor PTKs consist of an SH3 and an SH2 domain, a catalytic domain and a long carboxyl-terminal sequence containing nuclear localization and export signals, DNA and actin binding domains and several proline-rich regions. They are implicated in the regulation of growth and differentiation signals as well as cell adhesion [145]. Even though Abl kinases can directly bind to Cbl [146], their interaction is largely increased upon expression of ArgBP2, an adaptor protein containing three SH3 domains [41]. Cbl phosphorylation stabilizes the Cbl/Abl/ArgBP2 complex leading to enhanced Cbl-mediated ubiquitination and degradation of Abl and ArgBP2 [41]. Conversely, another adaptor protein, Nck, inhibits binding and phosphorylation of Cbl and Abl, probably through competitive interaction of its SH2 domain [51]. Cbl and Abl family kinases together with Nck are also implicated in signals responsible for the patterning of *Xenopus* embryos. [147]. Cbl was shown to inhibit anchorage-independent growth of v-Abl-transformed fibroblasts and to induce cell adhesion and spreading [148]. Cbl phosphorylation and interaction with CrkL and p85 PI-3 kinase were necessary for producing these effects [148]. Analogously, the BCR/Abl oncogene induces tyrosine phosphorylation of Cbl and formation of a

multiprotein complex containing BCR/Abl, Cbl, CrkL and PI-3 kinase [149, 150].

Cbl regulation of insulin functions

Insulin stimulation of glucose uptake in adipose and muscle tissues occurs via two signaling pathways that induce the translocation of the glucose transporter GLUT-4 to the plasma membrane. Both pathways are initiated by activation of the insulin receptor (IR). While the classical pathway is dependent on PI-3 kinase activity, the second pathway utilizes Cbl signaling from lipid rafts (reviewed in [151]). Cbl is rapidly tyrosine phosphorylated by IR kinase in 3T3L1 adipocytes, but not in fibroblasts or CHO cells overexpressing both IR and Cbl [152]. The IR does not directly associate with Cbl, and efficient phosphorylation requires the presence of adipose- and muscle-specific adaptors such as APS [29]. In the IR-APS-Cbl complex, Cbl has been shown to facilitate monoubiquitination of the IR β subunit [153]. Whether the Cbl-mediated ubiquitination leads to internalization and/or downregulation of IR still remains to be determined.

The IR preferentially phosphorylates Cbl at the tyrosines 371, 700 and 774 [29]. The latter two phosphotyrosines serve as docking sites for the SH2 domain of CrkII, while one of the SH3 domains of Crk is constitutively associated with C3G protein [154]. C3G acts as a guanine nucleotide exchange factor for small G proteins Rap1 [155] and TC10 [156]. Importantly, the Cbl-CrkII-C3G complex is targeted to the detergent-resistant membrane (DRM) microdomains (lipid rafts and caveolae) by Cbl-associated protein CAP, which is expressed in differentiated adipocytes [157]. CAP contains a Sorbin homology (SoHo) domain that binds flotillin, a component of lipid rafts [158], while its SH3 domains can interact with Cbl and APS [29]. In adipocytes, TC10, a small GTPase homologous to Cdc42, is localized in caveolar structures, and it seems that the CAP-mediated translocation of the Cbl/CrkII/C3G complex is necessary for its activation. The GTP-bound TC10 may initiate modification of cortical actin and stimulate actin polymerization that is needed for redistribution of GLUT-4 from the intracellular tubulovesicular system to the cell surface [159]. However, exactly how TC10 regulates GLUT-4 transport is not fully understood.

Type 1 diabetes (insulin-dependent diabetes mellitus) is an autoimmune disease with a multifactorial etiology. Interestingly, Cbl-b has been recently characterized as a susceptibility gene for type I diabetes in rats. These rats developed diabetes with severe insulitis [160]. The proline-rich region of Cbl-b that is deleted in diabetic (KDP, Komeda diabetes prone) rats is required for interaction with multiple proteins, including Vav1 [57]. Interestingly, impairment of the Cbl/Vav pathway contributes to development of spontaneous autoimmunity characterized by infiltration of lymphocytes into multiple tissues [161], indicating that the inability of Cbl-b to inhibit the Vav1 signaling pathway may be implicated in the pathogenesis of diabetes.

Functions of Cbl proteins in the immune system

Cbl and Cbl-b proteins play critical roles in controlling different functions of the immune system. The importance of Cbl is evidenced by its involvement in signaling by a variety of receptors present on different immune cells, including the T cell receptor (TCR) [48], the B cell receptor (BCR) [50], the immunoglobulin Fcγ receptors [162], receptors for interleukin-2 (IL-2) [163], IL-3 [164], IL-4 [165], IL-7 [123], Fas [166], the cell surface antigen CD38 [167], CD16 [168], CD9 [169], CD43 [170] and Ly49D [171]. Molecular details of Cbl's role in these pathways have been extensively discussed in several recent reviews [127, 172].

The essential role of Cbl proteins in immune cells was demonstrated by the generation of mice deficient for Cbl [173, 174] or Cbl-b [161, 175]. The major phenotypes observed in these mice are related to the alteration of signaling pathways in thymocytes or mature T cells. Cbl-deficient mice display increased TCR signaling in thymocytes involving the upregulation of ZAP-70 kinase activity [173, 174], whereas Cbl-b deficient mice display altered TCR function in mature peripheral T cells via deregulation of Vav guanine nucleotide exchange factor activity [161, 175]. Cbl-deficient mice display alteration of growth in a variety of tissues that is consistent with the ability of Cbl to negatively regulate mitogenic signals. Thymocytes from Cbl–/– mice have enhanced TCR-induced tyrosine phosphorylation of proteins in the absence of CD4 coreceptor ligation. In these thymocytes, activation of ZAP-70 occurs independently of CD4 stimulation and Lck activation [173]. Together with the higher expression of both CD3 and CD4, this results in upregulation of the Ras MAPK pathway involved in thymic positive selection [174].

Cbl-b knockout mice displayed spontaneous or induced autoimmunity disorders. One of the Cbl-b-deficient mouse strains shows spontaneous autoimmunity characterized by infiltration of lymphocytes into multiple tissues [161], while the other strain shows no significant signs of autoimmunity under normal conditions but is highly susceptible to experimental autoimmune encephalomyelitis [175]. In Cbl-b–/– mice, T cell proliferation, IL-2 production and phosphorylation of Vav1 are uncoupled from the requirement for CD28 costimulation. The Cbl-b-null mutation can fully restore T-cell-dependent antibody responses in CD28–/– mice. In contrast to Cbl-deficient mice, Cbl-b–/– T cells showed normal sig-

naling via tyrosine kinases ZAP-70 and Lck, Ras/mitogen-activated kinases, phospholipase Cγ-1 and calcium mobilization. On the other hand, activation of Vav was significantly enhanced in Cbl-b–/– T cells [175]. One way that Cbl-b regulates Vav activation is by controlling PI-3 kinase activity through a ubiquitin-dependent but proteolysis-independent regulation of the p85 subunit of PI-3 kinase [176].

Most of Cbl and Cbl-b functions are redundant in vivo, as knockout mice for either Cbl or Cbl-b are viable, fertile, and display cell and tissues restricted phenotypes. On the other hand, Cbl/Cbl-b double knockout mice die early during embryogenesis [177]. The generation of double Cbl/Cbl-b knockout in T cells showed that these cells become hyperresponsive to TCR activation due to defects in downregulation of TCR after ligand engagement [177]. Curiously, major downstream signaling pathways of activated TCR were not enhanced by the lack of both Cbl members. Activation of MAP kinases and Vav were comparable to wild-type cells, and phosphorylation of PLCγ was even decreased in Cbl–/–Cbl-b–/– cells, but the duration of signal, such as phosphorylation of Erk1/2, was strongly enhanced [177]. Furthermore, the spike of calcium signal seen in wild-type cells in response to TCR stimulation is absent in Cbl–/–Cbl-b–/– cells where calcium mobilization increases gradually [177]. This is consistent with several studies described in preceding sections supporting the hypothesis that Cbl proteins bind some signaling proteins to favor their mutual interaction and activation.

Cbl regulation of cell morphology

Adhesion and motility of cells have key roles in normal physiology and disease. They both require the assembly and organization of actin microfilaments into specific morphological structures of the cell such as stress fibers, lamellipodia, filopodia or podosomes. The balance between the assembly and disassembly of these structures is controlled by multiple signals. Cbl proteins have been implicated in regulation of actin dynamic induced by various factors.

Adhesion of macrophages induces tyrosine phosphorylation of Cbl as well as its translocation to the plasma membrane [143, 144]. Cbl is associated with SFKs (Hck, Fgr and Lyn), which mediate its phosphorylation, and together with PI-3 kinase activity, this appears to be critical in regulating actin cytoskeletal rearrangements that lead to cell spreading [144]. Blocking the activity of these kinases or depleting the cells of Cbl results in a blockage of cell spreading [144]. Furthermore, adhesion-induced phosphorylation of Cbl is suppressed when reorganization of the actin cytoskeleton is blocked [143]. Cbl localization in phalloidin-stained actin structures requires Cbl effectors bound to its carboxyl terminus, and deletion of these sites in Cbl inhibits the formation of actin lamellipodia, and membrane ruffles in fibroblasts during cell spreading and migration [178]. In addition, Cbl appears to regulate the dynamics of the actin cytoskeleton by interacting with the adaptor protein CAP, which also binds to focal adhesion kinase, and regulates the formation of stress fibers and focal adhesions in fibroblasts [179]. As we previously described, CAP plays an essential role in insulin-induced GLUT4 translocation to the plasma membrane by recruiting Cbl to lipid raft microdomains. Interestingly, disruption or stabilization of the cortical actin of adipocytes also results in inhibition of GLUT4 translocation and subsequent glucose uptake [159]. Therefore, Cbl might also be involved in control of the actin cytoskeleton dynamics necessary for the normal function of adipocytes. Similarly, a complex between Cbl and the adaptor protein CMS [44] is found at lamellipodia and the leading edges of cells. CMS has been shown to control actin reorganization via its interactions with p130Cas and SFKs [180].

The regulation of the actin cytoskeleton by Cbl and Src essential for the normal function of osteoclasts. This was first evidenced in mice deficient for Src kinase, which develop osteopetrosis due to a deficiency in bone resorption by osteoclasts [142]. Tyrosine phosphorylation of Cbl and its localization in a peripheral ring-like organization of podosomes are impaired in resorption-deficient Src–/– osteoclasts. Both Cbl and Src antisense oligonucleotides inhibit in vitro bone resorption by osteoclast-like cells [142]. Plating of normal osteoclasts onto vitronectin leads to formation of podosomes and increased cell motility. These two phenotypes were absent in Src–/– cells, while migration of osteoclasts was minimally reduced and no alterations of the bone development were observed in Cbl-deficient mice [173]. In another study, the absence of Cbl in osteoclasts led to a decrease in lamellipodia formation and cell migration [21].

In addition to spreading, Cbl is also involved in regulation of the migration of macrophages. Intergrin ligation by fibronectin in macrophages leads to tyrosine phosphorylation of Cbl and its association with SFKs Fgr and Lyn. In triple SFK-deficient Hck–/–Fgr–/–Lyn–/– cells, which are defective in spreading and migration in vivo, Cbl tyrosine phosphorylation is blocked and Cbl translocation to the membrane, together with Cbl-associated PI-3 kinase activity, is reduced [144].

Interestingly, very recent findings showed that Cbl may act as a negative regulator of actin polymerization induced by RTKs [181]. Expression of a truncated Cbl protein containing only the PTB and RING finger domains, which acts as a dominant-negative protein for the functions of Cbl associated with its carboxyl-terminal tail, leads to an increase in actin ruffle assembly in response to PDGF stimulation [181].

Cellular inhibitors of Cbl function

Cbl is subjected to numerous posttranslational modifications, including phosphorylation and ubiquitination, as well as interactions with multiple effectors in order to either promote or inhibit cellular signaling [73, 182]. Recently, several reports have described cellular inhibitors of Cbl functions that exert cell-specific effects on RTK signaling (fig. 5).

The family of Sprouty (Spry) proteins was first identified in *Drosophila* as a negative regulator of signaling by inhibiting the Ras signaling pathway, including EGFR and FGFR signaling [183, 184]. Four mammalian orthologues of *Drosophila* Sprouty (mSprouty1–4) were cloned and showed to be involved in both positive and negative receptor signaling [73, 185]. The conserved carboxyl terminus of Spry2 is responsible for the inhibition of EGF-induced MAP kinase activation, whereas the amino terminus enhances EGFR signaling by inhibiting Cbl-induced downregulation of activated EGFR [186]. Spry2 was found to interact with the RING finger domain of Cbl, thus blocking Cbl-directed ubiquitination and degradation of activated EGFR [32]. This inhibition of receptor downregulation results in potentiation of EGFR-mediated MAP kinase activation and ligand-dependent differentiation of PC12 cells [31, 186]. In addition, recent findings have shown that the interaction between Cbl and Spry2 is mediated by binding of the PTB domain of Cbl to the Y55 of Spry2 phosphorylated by the activated receptor [30]. Cbl then mediates the polyubiquitination of Spry2, which is followed by its proteasomal degradation [30, 187]. Thus, by removing Cbl from the activated RTK complexes, Spry2 inhibits its ability to downregulate RTKs [30, 187].

Tyrosine phosphorylation of Cbl is critical for interactions with a variety of SH2 domain-containing proteins. Therefore, dephosphorylation of Cbl, via the action of tyrosine phosphatases, is potentially an effective mechanism to block its functions. The treatment of cells with oxygen peroxide or pervanadate, two potent inhibitors of tyrosine phosphatases, leads to an increased tyrosine phosphorylation of Cbl [188]. This demonstrates that Cbl is indeed the substrate for tyrosine phosphatases and that its level of phosphorylation is dynamically regulated in cells. Furthermore, Cbl interacts with and is a substrate for several tyrosine phosphatases, including SHP-1, SHP-2 and Lyp1 [58, 189, 190]. SHP-1 can block the Fcγ-induced phagocytosis by macrophages [189]. The proposed mechanism includes the dephosphorylation of Cbl that can no longer interact with CrkL and is unable to activate Rac and PI-3 kinase. On the other hand, SHP-2 mediated dephosphorylation of Cbl leads to an increase in SDF-1 (Stromal cell-derived factor)-induced chemotaxis [58]. The tyrosine phosphatase Lyp1, predominantly expressed in lymphoid tissues, was found to be constitutively associated with Cbl in T cells [190], and overexpression of Lyp1 led to a decrease in tyrosine phosphorylation of Cbl, suggesting that Lyp1 may also regulate the association of Cbl with its SH2 binding partners.

Cbl is also phosphorylated on serine residues, a modification that is involved in the association of Cbl with 14-3-3 proteins. Interestingly, it seems that there is a competition between tyrosine and serine phosphorylation. PKCα and PKCτ can interact directly with Cbl, and serine phosphorylation of Cbl by PMA treatment of the cells results in reduction of tyrosine phosphorylation of Cbl and decreased association with PI-3 kinase and CrkL [191]. This finding suggests that serine phosphorylation of Cbl has inhibitory effects on its functions.

Cbl in oncogenic signaling networks

Cbl can be converted to an oncogenic protein by deletions or mutations that disturb its ability to downregulate RTKs (fig. 6). The v-Cbl protein, a truncated from of Cbl containing only the PTB domain, associates with EGFRs but is impaired in receptor ubiquitination and degradation,

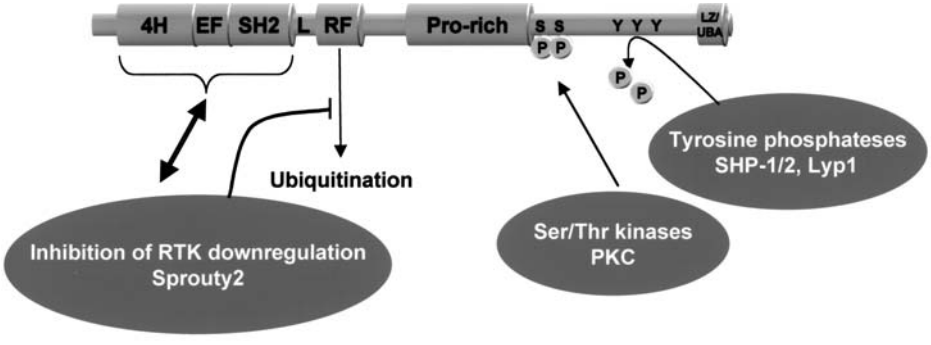

Figure 5. Inhibitors of Cbl functions. Sprouty2 inhibits Cbl-mediated downregulatoin of RTKs by blocking RING-dependent ubiquitination or by sequestering Cbl away from activated RTKs due to the binding of PTB domain to phosphorylated Y55 on Sprouty2. Tyrosine phosphatases SHP-1, SHP-2 and Lyp1 can dephosphorylate Cbl and disable binding of SH2 domain-containing partners, thus negatively regulating Cbl functions. Serine phosphorylation of Cbl by PKC inhibits phosphorylation of Cbl and its association with multiple effectors.

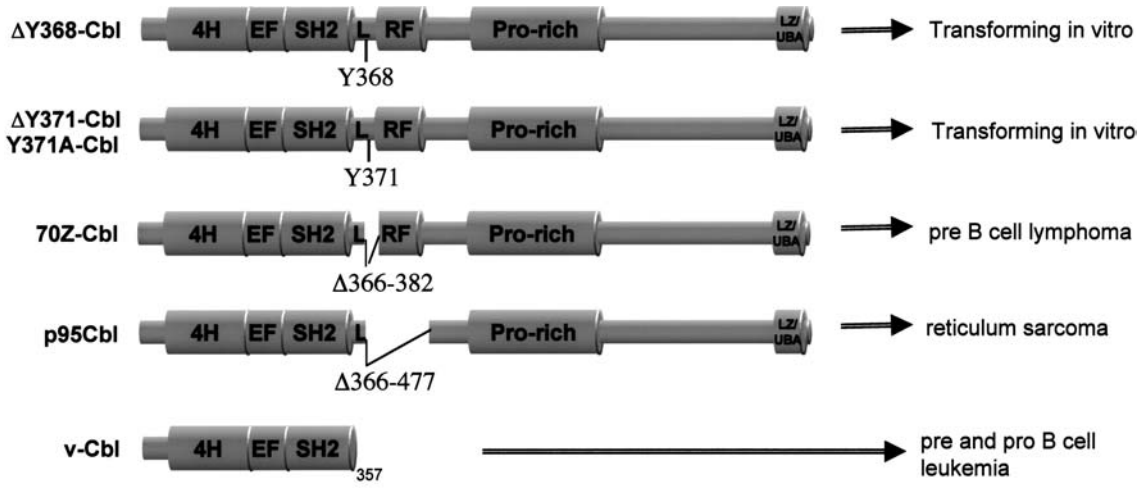

Figure 6. Oncogenic forms of Cbl possess a functional PTB domain and truncations or mutations involving linker region and RING finger. Expression of Cbl mutants in various tumor cell types is indicated on the right.

thus contributing to enhanced signaling and oncogenic transformation [66]. Accordingly a point mutation within the PTB domain (G306E) is able to abrogate the oncogenic potential of v-Cbl [26, 27]. It was proposed that the transforming properties of v-Cbl are due to competition with wild-type protein for binding to PTB domain effectors and preventing the normal function of endogenous Cbl. Similarly, other transforming forms of Cbl act in a dominant-negative fashion, and in all studied cases they bear deletions or mutations within RING finger and/or the preceding linker region. However, some mutations of the RING finger domain in the context of full-length Cbl are not sufficient to promote transformation, even though they abolish both Cbl-directed ubiquitination and downregulation of EGFR [192]. The linker region adjacent to the RING finger of Cbl was shown to be important for oncogenic transformation in cultured cells [15, 192]. Deletion of the Y368 residue that lies in the linker region has a dual effect – loss of EGFR ubiquitination and cell transformation – while deletion of C381 in the RING finger results only in a ubiquitination defect [192]. Moreover, deletion or mutation of tyrosine Y371 to alanine, but not mutation to phenylalanine, induces Cbl oncogenicity, probably by disrupting the structure of the linker region [146, 192]. Naturally occurring oncogenic mutant 70Z-Cbl, isolated from the 70Z/3 mouse pre-B cell lymphoma, has a deletion of 17 amino acids spanning the linker region and the beginning of the RING finger (mouse residues 366–382) [146], while p95Cbl from murine reticulum sarcoma cell line J-774 has a deletion of 111 amino acids, including the RING finger domain (residues 366–477) [193]. NIH3T3 cells expressing 70Z mutant, but not v-Cbl, showed a 10-fold increase in EGFR kinase activity in serum-starved cells and upon ligand stimulation [194]. In general, full-size Cbl bearing the 70Z deletion exerts more pronounced effects than v-Cbl in terms of activation of tyrosine kinases and recruitment of signaling complexes to the activated receptors [27, 146, 194]. Probably, while v-Cbl acts exclusively as a dominant-negative inhibitor, 70Z-Cbl functions additionally in a positive manner, interacting with a variety of Cbl partners. Transformation of NIH3T3 fibroblasts with 70Z-Cbl leads to an increase in tyrosine phosphorylation of endogenous cellular substrates, constitutive recruitment of the downstream components to the hyperphosphorylated PDGFRα and enhanced cell proliferation [27].

Numerous oncogenic RTKs were found to be defective in Cbl-mediated downregulatory pathways. Several strains of the avian erythroblastosis virus encode oncogenic orthologues of EGFR with deletions of a Cbl-docking site and thus escape targeting for endosomal degradation [17, 195]. Similarly, the Cbl binding site is missing in oncogenic forms of human EGFR found in brain tumors [196], the oncogenic form of HGFR (Trp-Met) [25] and in several chimeric TrkC receptors found in congenital sarcoma and acute myeloid leukemia [197]. In addition, Cbl has been recently shown to directly associate with a specific phosphotyrosine residue at the carboxyl terminus of feline c-Fms (CSF-1 receptor), which is absent in retroviral oncogene v-Fms [106] and mutated in patients with myelodysplasia and secondary acute myeloblastic leukemia [198, 199]. The above examples provide strong evidence that uncoupling Cbl from RTKs results in defective regulation of receptors activity and oncogenic transformation.

Oncogenic forms of Cbl are not only involved in cell growth but also in cell survival, invasion and metastasis. Cells expressing the oncogenic Cbl mutant ΔY371 have an increased level of Bcl-2 protein and are protected from induced apoptosis [200]. Intriguingly, the nuclear appearance of CARP 90 (Cbl apoptosis-related protein of 90 kDa), a possible alternatively spliced form of Cbl, has

been correlated with induction of apoptosis [201]. Some studies have suggested that cellular transformation by oncogenic forms of Cbl occurs by the activation of integrin-dependent, rather than growth factor-dependent pathway [202]. Indeed, ectopic expression of these mutants transforms NIH3T3 fibroblasts and results in anchorage-independent but serum-dependent growth [193, 202]. In addition, overexpression of 70Z-Cbl in epithelial MDCK cells induces reorganization of focal adhesion complexes and actin (i.e. there is a decrease in cortical actin and an increase in stress fibres), disruption of adherent junctions and cell dispersal [203]. Thus, signals mediated by Cbl downstream of integrins and RTKs might contribute to tumor progression.

Cbl functions as potential therapeutic targets

Development of human cancers is often associated with excessive RTK signaling that leads to tumor growth, metastasis and angiogenesis. Thus, controlling the degradation of oncogenic RTKs in primary tumors should, in principal, be a promising tool for anticancer therapies [204, 205]. Several types of antitumorigenic antibodies have been designed to inactivate oncogenic receptors in tumors. Monoclonal antibodies utilize multiple pathways to block oncogenic RTK functions, including inhibition of ligand binding, induction of receptor downregulation, as well as stimulation of differentiation or programmed cell death in tumor cells. Overexpression or the constitutive activity of ErbB family receptors is frequently detected in several types of cancer, and receptor amplification has been associated with a poor clinical prognosis [206]. Treatment with monoclonal antibodies against EGFR together with chemotherapy and radiotherapy efficiently blocked the development of established tumors [207]. Amplification or upregulation of ErbB-2, found in 25–30% of human breast, lung, prostate and gastric cancers, correlates with more aggressive subtypes of the disease [208, 209]. Most of the tumor-inhibitory antibodies induce accelerated clearance of ErbB-2 homodimers from the cell surface, while others inhibit the formation of heterodimers with other members of the ErbB family [210]. While a number of previous studies questioned Cbl association with ErbB-2, a recent report demonstrated the importance of Y1112 residue in mediating an interaction with the Cbl PTB domain [211]. Mutation of this putative Cbl-docking site blocks antibody-induced receptor internalization and degradation [211]. Tumorigenic ErbB-2 (Neu oncogene) is relatively weakly associated with Cbl and forms a ternary complex that includes Cbl, PI-3 kinase and Shc [212]. A recombinant humanized monoclonal antibody (Herceptin/Trastuzumab) that targets the extracellular domain of ErbB-2 has been successfully used in the treatment of metastatic breast cancer [213, 214]. While no effect is observed on cells with physiological expression of ErbB-2, interaction of anti-ErbB-2 antibodies with overexpressed receptors results in tyrosine phosphorylation of the RTK, internalization of the antibody-receptor complex and eventually a decrease of cell proliferation [215]. The effectiveness of Herceptin and other tumor-inhibiting antibodies is at least partially dependent on enhancing Cbl-mediated ErbB ubiquitination and downregulation [211]. The tumor-inhibitory antibodies that utilize the Cbl-downregulatory pathway require kinase activity and tyrosine phosphorylation at the C-terminal tails of ErbB proteins and, analogously to ligand-induced endocytosis, target only the membrane fraction of the receptors [211, 212]. Conversely, the use of specific tyrosine kinase inhibitors or the benzoquinoid ansamycin antibiotics geldanamycin or herbimycin A directs ErbB-2 to a degradative pathway mediated by chaperones and leads to proteasomal degradation of receptors both at the plasma membrane and endoplasmic reticulum [216, 217]. It is worth noting that combination of the two approaches has antagonistic, rather than additive or synergistic, effects on ErbB-2 downregulation [217] that may indicate competition within a common biochemical pathway.

Decreased levels of Cbl have been reported in early stages of murine immunodeficiency syndrome (M-AIDS) [218]. Infection with the RadLV-Rs murine leukemia virus leads to polyclonal proliferation and impaired responsiveness of T and B cells in lymph nodes. Reduced Cbl expression, observed both in T and B cells from lymph nodes but not from thymus of infected animals, was an effect of posttranslational regulation, since the Cbl mRNA level was not affected [218]. It has been proposed that the loss of Cbl is involved in initiation of a hyperproliferative process and is not required to sustain excessive cell growth [218]. In addition, peripheral T cells from human immunodeficiency virus-1 (HIV-1) positive patients have normal levels of Cbl, but largely increased levels of Cbl-b [219]. Constitutively high levels of Cbl-b correlate with lower ERK-1/2 phosphorylation and with a decreased proliferation in response to TCR stimulation [219]. The anergy observed in Cbl-b-overexpressing T cells correlates well with the hypersensitivity of Cbl-b–/– lymphocytes. Infection of B cells with the Epstein-Barr virus (EBV) also suppresses signaling from antigen receptor. In lymphoblastoid cell lines latently infected with EBV, the ligand-independent phosphorylation of Cbl leads to formation of a constitutive complex with phosphorylated BLNK, CrkL and C3G, and inhibition of PLC-γ2 activity [220]. These results indicate that the Cbl protein family contributes in multiple ways to persistent immune activation and decreased responsiveness caused by viral infection.

Clearly, better understanding of normal and deregulated Cbl functions could lead to development of novel and im-

proved therapeutic approaches that might be efficient in controlling disease progression, including infection and cancer.

Conclusions and future perspectives

Since its discovery in 1989, Cbl and the other members of the Cbl family have been primarily implicated in signal transduction by their ability to form complexes with a large variety of signaling molecules. A second wave of thinking, initiated by studies on SLI-1 in worm development, revealed the negative regulatory role of Cbl in tyrosine kinase-mediated signals. This was strongly supported by the generation of Cbl and Cbl-b deficient mice and by demonstrations that the RING finger domain of Cbl exerts ubiquitin ligase activity that is critical for RTK downregulation and degradation. Thus, it seems that Cbl proteins are endowed with both adapting and ubiquitin-ligating functions that may play different roles in cellular signaling. Current efforts are focused on understanding how these Cbl functions cooperate in the regulation of physiological and pathological processes in individual cells, tissues and living organisms. In order to understand these processes, we will need first to catalogue all proteins which interact with Cbl in particular cells at a given time or space and how they change or move within cells in response to changes of the environment. In addition, exciting and intriguing results are also expected from analysis of conditional mice depleted of Cbl and Cbl-b in numerous cell types. This will lead to the revelation of both shared and specific functions of these members of the Cbl family in vivo. Up to date, very little is known about functions of Cbl-c, the most divergent member of the Cbl family, which may exert biological properties uncommon to Cbl and Cbl-b.

It is now obvious that different ways of conjugating ubiquitin molecules to a substrate exist in cells, including polyubiquitination, monoubiquitination and multiubiquitination (multiple monoubiquitination) and that they are implicated in the control of various cellular processes. Does Cbl mediate mono- or polyubiquitination of the same or different targets in distinct cellular compartments? What are the regulatory mechanisms that control the ability of Cbl to catalyze diverse ubiquitin modifications on the target proteins, and what is the functional consequence of their ubiquitination in vivo? Further investigation of the role of Cbl in these as well as other processes will be a challenge for future research on Cbl signaling networks.

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